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PITTSBURGH UNIV PA DEPT OF PATHOLOGY  
MECHANISM OF ACTION OF ANTIGENS.(U)  
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6 MECHANISM OF ACTION OF ANTIGENS.

9 ANNUAL PROGRESS REPORT. (Final),

10 THOMAS J. GILL, III  
DEPARTMENT OF PATHOLOGY  
SCHOOL OF MEDICINE  
UNIVERSITY OF PITTSBURGH

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## SUMMARY

During this final year of support of this research project on the mechanism of action of antigens, very significant progress on several fronts has been made. The results have met our expectations of this project and have clearly defined the significant questions for final resolution by future research in this area.

→ A major advance has been the quantitation of IgG and IgM immunoglobulins present in the plasma membranes of thymic lymphocytes which have been considered until now to lack membrane immunoglobulin. The import of this finding is that the evidence for thymic lymphocytes as antigen-reactive cells is consistent with the postulate of immunoglobulin as cell receptor for antigen. Evidence for surface immunoglobulin on thymic lymphocytes was lacking because of a) the failure of surface iodination to label more than a small fraction of thymic lymphocyte immunoglobulin and b) the markedly greater efficiency of iodination of peripheral lymphocytes compared to thymic lymphocytes. Thymic lymphocyte membranes are not as efficiently labelled as membranes from splenic lymphocytes and more specifically, IgG immunoglobulin in thymic lymphocytes is not labelled at all. *The investigation concludes* We have concluded that IgG immunoglobulins are buried within the matrix of the plasma membrane and not accessible to external labelling reagents. *He has also*

During the past year we have selectively extracted thymic lymphocyte glycoproteins using the lithium diiodosalicylate method. This method of extraction has been shown to be highly efficient for glycoproteins in particular and recent results have demonstrated that thymus specific antigen is enriched in these extracts. The extracted glycoproteins have been characterized and the method has been established as an efficient reproducible technique for the isolation and characterization of the rat lymphocyte antigens which are clearly associated with genetic control of immune responsiveness.

→ The delineation of differences between high responder and low responder lymphocytes is now being studied by in vitro biosynthesis of lymphocyte membrane components using incorporation of radioactive amino acids. We have found in thymus lymphocytes that two proteins of approximately 30,000 and 15,000 daltons rapidly incorporate amino acids and have a half-life of approximately 6 hours. These proteins are being identified by immunochemical methods already developed in the laboratory. The rates of synthesis and degradation of the thymocyte membrane IgG immunoglobulin are also being compared in high and low responder strains to define metabolic differences which correlate with in vivo immunological responsiveness. Whether or not these buried immunoglobulins function as antigen receptors requires labelling of this immunoglobulin to a very high specific activity with radioactive amino acids. Final resolution of this question will rest on future work based on this fundamental work supported by the Research and Development Command.

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## FOREWORD

In conducting the research described herein, the investigators adhered to the "Guide for Laboratory Facilities and Care" as promulgated by the Committee in the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

### Genetically regulated differences in lymphocyte membranes

Since the previous studies on the structure of lymphocyte membranes have shown so far that there are major differences between thymus and spleen lymphocyte membranes but no strain or sex variations which can be correlated with the responder status, we have postulated that there are genetic differences in the rates of synthesis and degradation of the antigen receptors between high and low responder strains. For the last six months we have established methodology for the determination of rates of synthesis and degradation of membrane proteins in thymic and splenic lymphocytes. These in vitro methods have been tested with mitogen-stimulated thymic lymphocytes and we have determined the optimal conditions for cultivation and preliminary results for two proteins.

Within one hour thymic lymphocytes incorporate radioactive amino acid precursors into two proteins of molecular weights of approximately 30,000 and 15,000 daltons. These proteins can be labelled with various radioactive amino acids including  $^3\text{H}$  aspartic acid  $^3\text{H}$ -leucine and  $^3\text{H}$ -serine. We have determined that the half-life of disappearance of these proteins from thymic lymphocytes is approximately 6 hours. This is consistent with the half-life of histocompatibility antigens in mouse and man as well as of rapidly synthesized and secreted immunoglobulins. The current studies have established the methods for comparison between high and low responder lymphocytes from thymus, spleen and peripheral lymph nodes. These in vitro methods developed during this final year of support have provided two vantage points for the extension of our previous work to the ultimate definition of the structural basis of the immunological differences between high and low responders. First, development of in vitro methods for study of turnover of specific membrane proteins has provided a technique for the measurement of metabolic differences between membrane proteins in high and low responder lymphoid cells. Secondly, the radioactive labelling of membrane proteins to a very high specific activity has provided a means for the ultimate isolation and characterization of antigen receptors, for it will be possible by tracer methods to fractionate minute quantities in sufficient yield to analyze them by polyacrylamide gel electrophoresis and immunochemical means.

The in vitro incorporation studies initiated during this past year will also enable systematic investigation of histocompatibility antigens of the rat which are associated with immunological responsiveness. The specific antisera available against the various histocompatibility antigens of high and low responder strains, are being used to characterize these different histocompatibility antigens by a variety of techniques already developed in the laboratory. Previous studies with solubilized erythrocyte membranes have given variable results. Our current approach is to extract glycoproteins from the lymphocyte surface using the lithium diiodosalicylate which extracts from 4-6% of all the membrane proteins but selectively extracts 40-60% of lymphocyte carbohydrates. Since all lymphocyte membrane glycoproteins are extracted in proportion to their concentration in the isolated membranes, this method has been adapted to the isolation and characterization of rat lymphocyte membrane glycoproteins.

### Comparison of thymic and splenic leukocyte membranes

Previous results had shown that the major difference between thymic and splenic lymphocyte membranes was the difference in membrane glycoproteins. Specifically the thymus glycoprotein of 27,000-28,000 daltons was identified not only in isolated membranes but has been extracted in good yield by lithium diiodosalicylate (LIS). The results are that LIS



extracts consist of highly enriched membrane glycoproteins, and the polyacrylamide gel electrophoretic patterns of LIS extracted proteins have shown not only all membrane glycoproteins in approximately the same proportions as in isolated membrane, but also the major thymus specific glycoprotein. When tested against specific antithymocyte antisera, antigenic activity is enriched several fold in the LIS extract. We have demonstrated previously that the precipitate obtained from incubation of detergent-solubilized membranes with antithymocyte antisera contained two proteins of approximately 33,000 and 27,000 daltons. From these data we earlier concluded that antithymocyte antisera identified at least 2 different thymocyte membrane proteins. Recent experiments with immunoprecipitation of LIS extracted glycoproteins shows these two membrane proteins as well.

The differences between thymic and splenic lymphocyte membranes has also been studied using specific antisera for immunoglobulin chains. Immunofluorescence studies with antiserum specific for heavy chains of IgG immunoglobulin have been carried out under a variety of conditions in order to detect low levels of surface IgG immunoglobulins. These studies have failed to detect any external membrane IgG immunoglobulin and these results with thymocytes are consistent with our previous results indicating that this immunoglobulin comprises about 1% of the membrane protein but is largely buried within the matrix or on the interior surface of the membrane.

#### Turnover of membrane proteins, antigens and receptors

A major part of the research activity during the last several months has been the development of methodology for the study of biosynthesis of membrane immunoglobulins, antigens and antigen receptors. A system has been developed using radioactive aspartic acid, leucine or serine for purposes of studying rates of incorporation and degradation of surface proteins. We have found that radioactive serine is rapidly incorporated into proteins to give relatively high specific activity in a short incubation period and this amino acid is not reutilized rapidly by lymphoid cells. Using  $10^8$  to  $10^9$  thymic or splenic lymphocytes in basal medium Eagle's tissue culture medium incubated over a period of 1 to 16 hours, we have been able to identify two rapidly synthesized proteins of approximately 30,000 and 15,000 daltons. These two proteins have been analyzed by polyacrylamide gel electrophoresis and their rates of synthesis and degradation are being studied. The rate of disappearance from thymic lymphocytes is in approximately six hours, a finding consistent with the turnover rates of H-2 histocompatibility antigens of the mouse and HL-A antigens in man. During this past year we have also obtained the specific antisera against light chain, gamma chain, chain of rat immunoglobulins, as well as antisera against whole immunoglobulins including IgG, IgM, IgA, and IgE. This study has been initiated to study the rates of synthesis of each one of these immunoglobulins by thymic as well as splenic lymphocytes in high and low responder animals, and the method of detection has been already developed. This detection technique depends upon acid urea extraction, and concentration of cytoplasmic, nuclear and secreted proteins for analysis by polyacrylamide gel electrophoresis and two dimensional electrophoresis of the proteins into antibody-containing agarose. For solubilized membrane proteins, this analysis required a modification of the two-dimensional immuno-electrophoresis method of Laurell, and this has already been developed in our laboratory according to the method of Converse and Papermaster.

Thus, the progress in this research program has evolved from the recognition that there are virtually no compositional, enzymatic, or structural differences between high and low responder lymphocyte membranes, but there are major differences between thymus and peripheral lymphoid cells. From these earlier investigations, it appears that mechanism of action of antigen involves metabolic differences between high and low responder lymphoid cells, particularly metabolic differences affecting membrane immunoglobulin and receptors.

Therefore, the approach to the mechanism of action of antigen has evolved into the study of turnover of lymphocyte membrane proteins using techniques of *in vitro* radioactive amino acid incorporation, two dimensional immunoelectrophoresis of membrane proteins, autoradiography and liquid scintillation spectrometry. A major phase of structural biochemical studies of lymphocyte membrane proteins has been concluded, therefore, and we have entered the final phase in the study of the mechanism of action of antigens in regulating the immune response at the cellular level.



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